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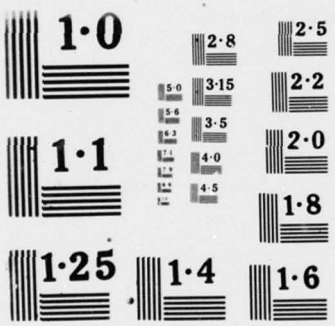

Studies of Viral Hepatitis,

Final Progress Report

A. W. Holmes, Principal Investigator
F. Deinhardt, Co-Investigator

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Introduction

Previous studies from our laboratories have reported the induction of serially transmissible viral hepatitis in marmoset monkeys (1), our ability to identify correctly specimens coded elsewhere and studied by us "blindly" (2,3), our observations of some of the physico-chemical properties of the causative agent or agents (4), and the neutralization of infectivity by human convalescent serum (5). These studies were carried out under contract DA 49-193-MD-2721 with the Research and Development Command and progress reports under that contract detailed those observations. Contract DADA 17-73-(3136) supported this work since 1 July 1973, but in June 1974 was approved only for terminal funding through December 1974.

Current Status of the Work

1. Serial passage of MS-1 in marmosets

Our earlier reports related the difficulties encountered in attempts to pass MS-1 infection in marmosets in series. Enhancement of infectivity by centrifugation of acute phase serums through cesium chloride density gradients, and selective inoculation of the gradient fraction between densities 1.19 and 1.23 gm/ml has enabled us to accomplish serial passage. At this time untreated acute phase serums from animals of the second to fourth passage in marmosets of MS-1 can be passed without the necessity for ultracentrifugation.

The passage series is illustrated in Table 1. In earlier experiments five different groups of marmosets had been inoculated with acute phase plasmas of human origin. (All of these inocula were obtained in the MS-1 volunteer studies at the Joliet State Prison, Joliet, Illinois.) In one experiment the inoculum was plasma from volunteer Kirk, obtained 30 days after inoculation and in the early acute phase of his hepatitis. In the other four the inoculum was an aliquot of fractions with densities ranging from 1.19-1.22 gm/ml obtained by centrifugation of a pool of acute phase human plasmas (the bulk of the plasma came from volunteer Miles, 36 days after inoculation) on a cesium chloride gradient. This particular density range was chosen on the basis of previous experiments with GB and MS-1 in which the infectious agent was shown to sediment at that level. A summary of all experiments in which serum fractions from cesium chloride gradients were inoculated into marmosets to assay infectivity is in Table 2. Generally one can say that the density of the agent or agents in the serum is in the range mentioned above, and those experiments in which fractions ranging in density from 1.23-1.45 gm/ml were studied were clearly negative.

Acute phase serum was harvested from the marmosets in the initial MS-1 experiments (first marmoset passage) at the time when elevations of serum hepatocellular enzyme activities first became detectable. These serums were pooled (total volume 7 ml) and this pool was centrifuged

through a cesium chloride gradient. The specifics of the method of centrifugation are in reference 5.) The fraction of density range 1.19-1.23 gm/ml from this centrifugation was used as the inoculum for the second passage. Both centrifuged and raw serum were used at the third passage, and it is apparent that infection was induced equally effectively with both materials. The fourth passage was accomplished by simple pooling of acute phase serums without any special treatment of the serum pool.

Table 1 shows that MS-1 infection has been established in serial passage in marmosets. As has been our experience with other human specimens, establishment in marmoset passage has been associated with some shortening of incubation period and enhanced infectivity, suggesting adaptation of the agent to the marmoset host. The third passage harvest has been titrated in marmosets and its infectivity by the intramuscular route is 1.2×10^5 MID_{50}/ml.

2. In vivo evaluation of immunity conferred by infection

In previous experiments we have examined the question of whether hepatitis caused in the marmoset by a given inoculum confers resistance to attempted reinfection with either the same infectious material or infectious serum derived originally from another source. Tables 3 through 6 summarize various experiments testing homologous and heterologous resistance to challenge inoculation with MS-1 or GB. Homologous resistance (Tables 3 and 4) is apparent for each agent. There is also good evidence that prior infection with GB will protect against MS-1 challenge (Table 5). Conflicting results have been obtained in experiments in which animals previously infected with MS-1 have been challenged with GB (Table 6). In an early experiment previous hepatitis following inoculation with MS-1 did not protect marmosets from intravenous challenge inoculation with GB. Later, the second experiment in Table 6 was performed, and there appeared to be protection against intramuscular challenge, even though the challenge dose was much larger in terms of infectivity. This divergence of results needs to be explored further.

Another observation in these challenge experiments has been of the phenomenon we have termed the "transitory early response" (TER). This is characterized by the appearance of striking elevations of serum enzyme activities, and sometimes serum bilirubin concentration, occurring 6-16 days after challenge inoculation. These striking abnormalities are associated with impressive hepatocellular necrosis and infiltration of liver biopsy specimens with inflammatory cells, but these changes are of very short duration and revert entirely to normal within five to fifteen days. We have previously suggested (4) that this might represent some sort of hyperergic response, but we have no good evidence for this. Further study of this phenomenon is in order, as more animals become available.

3. Attempted neutralization experiments

We have reported neutralization of infectivity of acute phase human hepatitis A serum by convalescent human serum (5). We hope to extend these observations and demonstrate also neutralization by pooled human immune serum globulin, as has been shown by others (6). Two experiments have been carried out thus far, but unfortunately in each one problems arose to render the results inconclusive.

In the first case, infectious marmoset serum from the GB passage series was incubated with two different preparations of pooled human immune serum globulin. One of these was obtained from Connaught Medical Laboratories, Toronto, Ontario, Canada (Connaught), and the other from the Merck, Sharpe, and Dohme Laboratories (MSD). These globulin preparations had been obtained specifically because they had not had any preservative added, to exclude the possibility of chemical inactivation of infectivity. Aliquots of infectious marmoset serum from the GB passage series were incubated with equal amounts of Connaught and MSD globulin at 37°C for one hour and then at 4°C overnight. The mixtures were clarified by centrifugation at 10,000xG for 30 minutes and subsequently inoculated into groups of marmosets at a dose of 0.5ml intramuscularly per marmoset. Groups of animals were also inoculated with 0.25 ml of MSD globulin alone, and with 0.25 ml of the diluted marmoset passage serum alone as negative and positive controls, respectively. (An attempt was made in an earlier experiment to titrate the infectivity of the eleventh passage of the GB series, and the titer was found to be in excess of 10^6 MID_{50}/ml when the serum was inoculated by the intravenous route. Because we had not seen titers higher than this in previous titrations, we made the assumption that it would be appropriate to dilute the serum pool 10-4.)

The marmosets were observed by our usual protocol, with weekly bleedings and biweekly liver biopsies, and the results are summarized in Table 7. Clearly, at least two of the groups were mislabelled. It is our suspicion that the GB alone and GB + Connaught groups were mixed, but we cannot prove this, and the experiment must be repeated.

In the second case, an attempt was made to neutralize MS-1 marmoset passage material with gamma globulin. A preliminary titration of the MS-1 marmoset passage 3 pool had given an infectivity titer of 1.2×10^5 MID_{50}/ml by the intramuscular route. The dilution chosen for this experiment gave a dose per marmoset of about 1250 MID_{50}. Aliquots of the diluted infectious serum were incubated with an equal amount of convalescent serum from volunteer Miles (M-90) which had previously neutralized human MS-1, and with equal amounts of Connaught and MSD globulins. These mixtures were treated in the manner described in the previous paragraph and inoculated into groups of marmosets. Another group of marmosets was inoculated with the MS-1 pool alone as a positive control. The results of this study

are seen in Table 8. While M-90 plasma inhibited the infectivity of MS-1 passage serum, there was no evidence of neutralization by either Connaught or MSD globulin. We suspect that the infecting dose of MS-1 pool in this experiment was too large, thereby overriding the amount of antibody present, and we do not consider this a valid test for the capacity of either the Connaught or MSD globulins to neutralize MS-1.

The last experiment points again to the difficulties which we have encountered previously in neutralization tests performed in vivo. It is obvious that a slight overdose of virus can lead to failure of neutralization as measured by the detection of biochemical and morphological evidence of hepatitis. This has been discussed in the past, and may be due in part to the sensitivity of the host to small amounts of virus which have not been neutralized. Another factor which could enhance this apparent sensitivity would be the dissociation of some of the virus-antibody mixture after the small volume (0.5 ml) is inoculated into the comparatively large (25-30 ml) blood volume of the marmoset.

Experiments to reexamine this question have been designed.

4. Infectivity of human hepatitis A stool

Some studies have been carried out to assay the infectivity of fecal material from the Joliet volunteers. Table 9 summarizes these results. An attempt was made to define a correlation between the presence of virus-like particles as described by Feinstone, et al. (7), the presence of fecal antigen as described by Cross, et al. (8), and infectivity when inoculated into marmosets by the intramuscular route. While day 7 stool from volunteer Ferguson was negative in all tests, later samples did contain particles and antigen, and did cause hepatitis. These studies are thus far limited in their scope, but they suggest some correlation between these techniques which bears further exploration. They are important also, however, as they represent the first demonstration of infectivity of acute phase hepatitis A stool for marmosets. Other studies in our laboratories, funded by the Food and Drug Administration, deal with further exploration of the significance of the fecal antigen and its relationship to the agent or agents of hepatitis A.

5. Studies done in vitro

The experiments which have been carried out in vitro have been directed toward the antigenic identification of the agent or agents causing hepatitis A in marmosets. They can be classified by the kinds of techniques used. Some have not proved helpful and have been discarded, while others are still under active study.

A. Immune electron microscopy

We have collaborated with Dr. Norman Anderson, Oak Ridge National Laboratories, in attempts to identify a virus-like particle in serum specimens which have been proven infectious for marmosets, and to demonstrate agglutination of such particles by antibody in serums from humans or marmosets convalescent from hepatitis. Aliquots of the following specimens were subjected to ultracentrifugation on a cesium chloride gradient:

i. plasma from volunteer Kirk, 30 days after inoculation (K-30)

ii. plasma from volunteer Miles, 36 days after inoculation (M-36)

iii. acute phase marmoset serum, GB passage series, P_{11}(GB).

Fractions with densities from 1.12-1.25 gm/ml were harvested and examined in various experiments and no convincing virus particles were demonstrated with the exception of the following possibly suggestive observations:

i. The portions of the gradient prepared from K-30 at 1.18-1.22 and 1.22-1.25 gm/ml contained small numbers of particles 20-30 nm in diameter but not sufficiently distinct in their morphology to permit clear identification as virus particles. These particles were agglutinated by convalescent plasma from volunteers Kirk and Miles, (the Miles convalescent plasma has been shown to neutralize MS-1 infectivity in vivo). Preinoculation plasma from Kirk and Miles, normal marmoset serum, marmoset anti-GB serum and MSD gamma globulin (see Section 3) failed to agglutinate these particles.

ii. The fraction of the M-36 gradient at density 1.18-1.22 gm/ml contained similar particles, but there was no clear evidence of agglutination by either Kirk or Miles convalescent plasma, nor was their agglutination by Miles preinoculation plasma.

iii. The preparation of the same density from the GB serum also contained similar particles, but again there was no clear evidence for agglutination by any of the above mentioned materials.

The HB_SAG-anti-HB_S system was used as a control, and particles and immunoprecipitation were clearly demonstrated using the same methods. A major problem in the interpretation of these results stems from the fact that convalescent plasma was used, rendering the electron photomicrographs considerably less clear than would ordinarily be hoped. Some of these results are encouraging, however, and currently a cooperative study is under way with Drs. Almeida and Zuckerman in London.

B. Radioimmunoassay

The unquestioned success and sensitivity of radioimmunoassay procedures in detecting HB_SAg and anti-HB_S encouraged us to apply them in a search for specific hepatitis A antigens. A group of eight marmosets which previously had had hepatitis following GB inoculation were reinoculated and presumably "hyperimmune" serum was harvested from them. As yet this serum has not been tested in vivo. A portion of it has been fractionated to obtain the gamma globulin, and this globulin was labelled by the Chloramine-T method. Gamma globulin was prepared in parallel from anti-HB_S-containing human serum and was labelled with ^{125}I under identical conditions. HB_SAg was prepared by the method of Gerin, et al., (J. Virol. 4: 763, 1969). Various test methods were tried:

i. Differential precipitation of ag-ab complexes with sodium sulfate

ii. Solid phase radioimmunoassay in plastic tubes

a. tubes coated with immune or normal serum globulins and reaction with ^{125}I labelled fraction from cesium chloride gradients of infectious marmoset serum (GB) or HB_SAg

b. tubes coated with immune serum globulins and reacted with unlabelled fraction (as in a, above) or HB_SAg plus ^{125}I labelled gamma globulins of the respective immune serums.

iii. Solid phase radioimmunoassay with activated Sepharose. Tests were done both in colums and as Sepharose slurries with continuous shaking during the reaction time and extensive washing by centrifugation afterwards.

a. normal or immune serum globulins coupled to Sepharose and reaction with ^{125}I labelled fractions as in ii., a, above, or with HB_SAg.

b. unlabelled fraction (as in ii., a, above) or HB_SAg coupled to Sepharose and reaction with the respective ^{125}I labelled normal or immune serum globulins.

C. Hemagglutination

Formalinized or tannic acid treated guinea pig erythrocytes were coated with the infectious fraction (as in B, ii, a, above) and hemagglutination was sought with normal and immune serums. None was found.

While the tests listed under B and C above and performed with HB_SAg and anti-HB_S gave satisfactory results, no conclusive positive results were obtained with GB antigen and antibodies.

The failure of these initial attempts to develop a radioimmunoassay was probably due to a low concentration of antigen and/or antibody in the test samples. In addition, the antigen preparations still contained large amounts of contaminating proteins. In further experiments antigen preparations will be purified by sequential isopycnic and rate zonal centrifugation: further purification will be attempted by immunoadsorbent columns and by selective concentration of antigen and antibody complexes on a gradient interface. The latter technique has been worked out for HB_SAg-anti-HB_S complexes, and gives excellent results in that system with a high level of sensitivity. These experiments will also be performed in the future with MS-1 passage materials and serums proven to contain neutralizing antibodies, which were not available to us at the time of these initial tests.

D. Tests for specificity of the "fecal antigen"(8)

Dr. Geoffrey Cross is currently spending two years with us as a visiting scientist, and we have begun to evaluate further the specificity of his test system.

i. Under code, Dr. Purcell has found that the rabbit antisera which Dr. Cross has prepared will agglutinate the fecal particle described by Feinstone, et al. (7)

ii. In cooperative studies, Dr. Zuckerman in London has detected fecal antigen in 17 of 25 stools from patients in the acute phase of hepatitis A. Using Dr. Cross' rabbit antiserum and immune electron microscopy, the antigenicity is seen to be associated with 30 and occasionally 45 nm particles. Ten control stools were negative in this test system.

iii. In our laboratory Dr. Cross evaluated 12 fecal specimens sent him under code by Dr. Zuckerman. The results are tabulated in Table 10. In all but one case acute phase stool-was identified as either positive (5 samples) or questionable (2 samples). Each control stool was correctly identified as negative.

iv. Dr. Cross could detect material reacting with his antiserum in immunoelectroosmophoresis in marmoset stools during the acute phase of hepatitis, but not in about 800 control marmoset stool samples. Though the antigenic identity of the material reacting in

the positive marmoset stools with Dr. Cross' standard antigen from human stools has as yet been established, studies to determine this are currently being performed.

v. Dr. Cross' antiserum reacted with some but not with all human MS-1 volunteer stools (Table 9) which were proven infectious in marmosets.

Although these studies were promising, they need further controls and confirmation, and such studies are planned for the future.

Significance of Study

It is felt that the significance of these studies is self-evident. Viral hepatitis, type A. continues to be of concern to both military and civilian populations. Its control will not be secure until the causative agent or agents can be clearly identified and appropriate vaccines produced. Much progress has been made and it is reasonable to hope that through these kinds of studies positive control of hepatitis A will be available to us in the near future.

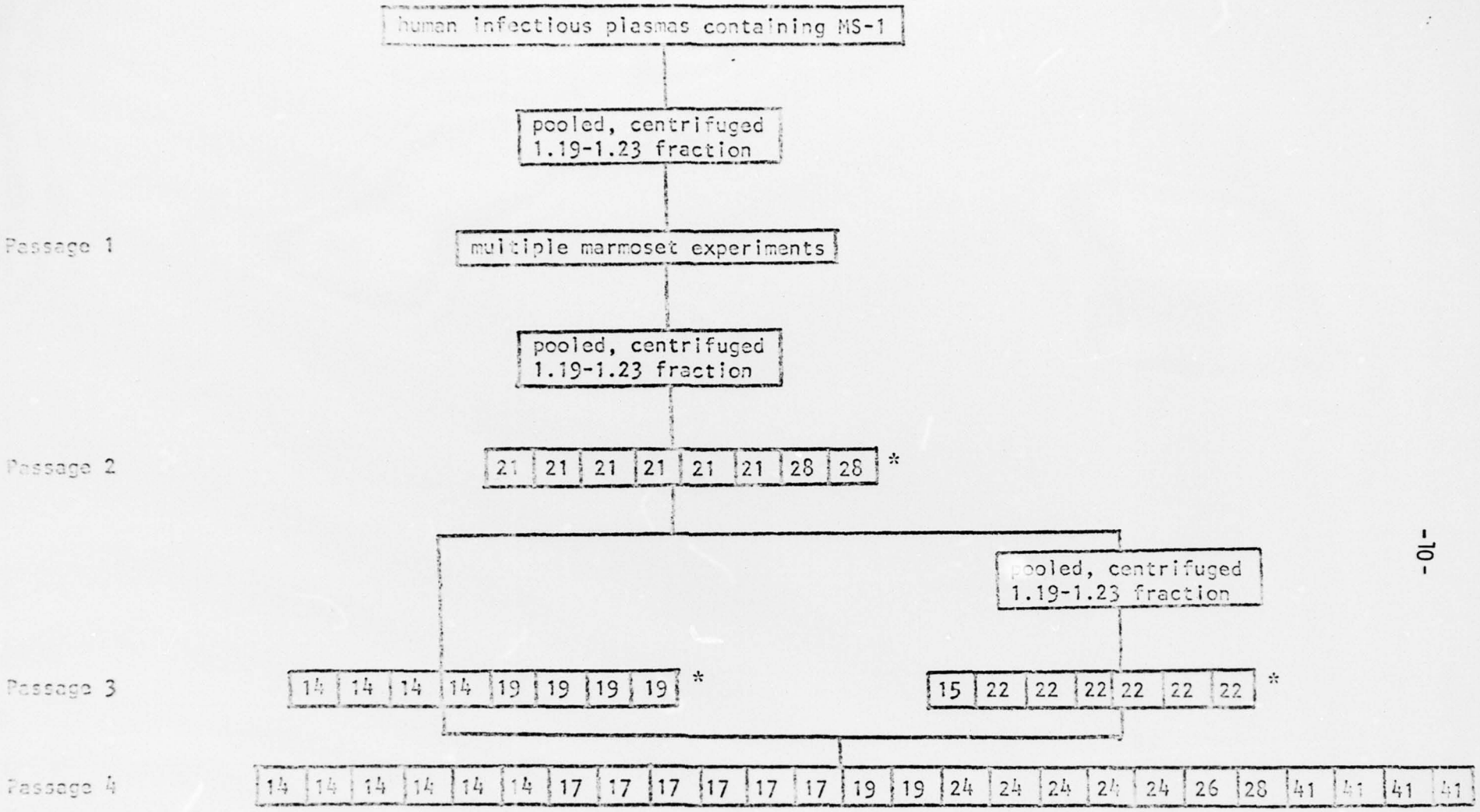

Table 1: MS-1 passage in marmosets.

*In these diagrams, each block represents a single marmoset. The number within the block indicates the incubation period in days.

Table 2. Cesium chloride gradient fractions which have been tested for infectivity in marmosets

Density	Source	Result	Marmosets with Hepatitis +	±	0
≤1.09	Barker*	+	3	1	0
≤1.18	MS-1(human)	+	3	0	3
1.18-1.20	MS-1(human	+	3	1	2
1.19	Barker	+	6	0	0
1.19-1.20	Barker	weak +	1	0	3
1.20	Barker	weak +	2	0	3
1.20-1.23	MS-1(human)	+	4	1	1
1.20-1.23	MS-1(marmoset)	+	8	0	0
1.20-1.23	MS-1(marmoset)	+	7	0	0
1.21	Barker	+	6	0	0
1.21	Barker	+	4	1	0
1.22-1.25	Barker	+	6	0	0
1.23-1.25	Barker	-	0	0	5
1.23-1.25 (includes pellet)	MS-1(human)	-	0	0	5
1.28	Barker	-	0	0	6
1.30	Barker	-	0	0	6
1.35-1.38	Barker	0	0	0	5
1.39-1.45	Barker	0	0	0	4

*all Barker samples are marmoset passage materials

Table 3: Results of MS-1 challenge of old MS-1

A. All animals had had their primary inoculation with human MS-1 plasma. They were challenged with human MS-1 plasma (acute phase, from volunteer Miles, day 36) intravenously. (Experiment H-219)

First MS-1 result	MS-1 challenge		
	+	±	-
+	0	0	3
±	0	0	2
-	0	0	1
control	3	1	1

B. Five animals had had primary inoculation with human MS-1 plasma, while four had had marmoset MS-1 passage serum. All were challenged with marmoset passage three of MS-1 intramuscularly, in a dose of about 1200 marmoset infectious units per animal. (Experiment H-255)

First MS-1 result	MS-1 challenge	
	+	-
+	1*	6
-	2	0
control	5	0

*Transitory early response(see text) This animal had been inoculated originally with plasma from volunteer Kirk, day 30

Table 4: Results of GB challenge of old GB

A. All but one marmoset had had primary inoculation with GB marmoset passage serum. The one exception had received original GB human serum. Challenge was with various doses of GB marmoset passage serum intravenously. (Experiments H-103, H-142, H-167)

First GB result	GB challenge		
	+	±	-
+	2*	0	12
±	0	0	1
-	0	0	1
controls	10	3	0

*transitory early response (TER)

B. All animals had had primary inoculation with various levels of GB marmoset passage serum. They were challenged with 0.5 ml of GB marmoset passage 11 (diluted 1:50 in HBSS) intramuscularly. (If given intravenously this would have represented over 32,000 marmoset infectious units) (Experiment H-254)

First GB result	GB challenge		
	+	±	-
+	1*	0	6
controls	4	0	0

*TER

Table 5: Results of MS-1 challenge of old GB

A. All marmosets had had primary inoculation with GB marmoset passage serum of various levels. They were challenged with acute phase (day 36) plasma from volunteer Miles, diluted 1:2 in HBSS, 0.5 ml intravenously. (Experiment H-219)

GB result	MS-1 challenge result		
	+	±	-
+	0	0	3
±	1	0	0
-	2	0	0
controls	3	1	1

B. All marmosets had had primary inoculation with GB marmoset passage serum of various levels. They were challenged with 1200 marmoset infectious units of the third passage of MS-1 in marmosets, given intramuscularly. (Experiment H-253)

GB result	MS-1 challenge result		
	+	±	-
+	0	1	6
controls	5	0	0

Table 6: Results of GB challenge of old MS-1

A. All marmosets had had primary inoculation with human MS-1 plasma. They were challenged with approximately 500 marmoset infectious units of the ninth passage of GB in marmosets, by intravenous inoculation. (Experiment H-157)

MS-1 result	GB challenge result		
	+	±	-
+	9	1	0
-	2	1	0
controls*	9	4	4

*controls for this experiment consisted of animals which were either previously uninoculated, or had been inoculated with plasmas drawn from the Joliet volunteers before their (the volunteers') inoculation with MS-1.

B. Nine animals were used, five of which had had primary inoculation with human MS-1 plasma and four of which had had primary inoculation with marmoset MS-1 passage serum. All were challenged with more than 32,000 marmoset infectious units of the eleventh passage of GB in marmosets. The challenge inoculum was given intramuscularly. (Experiment H-256)

MS-1 result	GB challenge result		
	+	±*	-
+	0	1	6
-	0	1	1
controls	4	0	0

*the two ± animals had originally been inoculated with human MS-1 material

Table 7: Neutralization of the GB agent by gamma globulin
(see text for particulars)

Inoculum	Inoculated	Hepatitis	±	Negative	Incubation period, days
GB alone	11	1	1	9	10,29
MSD* alone	8	0	0	8	-
GB + MSD*	12	9	0	3	22,22,30,30,3 30,44,44,44
GB + Connaught**	11	9	0	2	21,21,21,28,2 28,28,28,29

*pooled human gamma globulin obatined from Merck, Sharpe & Dohme Labs.

**pooled human gamma globulin obtained from Connaught Medical Laboratori

Table 8: Neutralization of MS-1 by gamma globulin
(see text for particulars)

Inoculum	Marmosets Inoculated	Hepatitis	±	Negative	Incubation period, days
MS-1 alone	6	5	1	0	8,15,15,29,29 29
MS-1 + MSD*	6	6	0	0	14,14,14,14, 21,21
MS-1 + Connaught**	6	6	0	0	14,14,21,21, 21 21
MS-1 + M-90***	6	2	2	2	14,28,28,28

*pooled human gamma globulin from Merck, Sharpe & Dohme Laboratories

**pooled human gamma globulin from Connaught Medical Laboratories

***convalescent plasma from volunteer Miles, 90 days after inoculation

Table 9: Determination of infectivity of human MS-1 stool for marmos

Volunteer	Day[1]	Particles[2]	Antigen[3]	Infectivity[4]
Ferguson	7	-	-	0/6 marmosets
	33	++++	+	2/6 marmosets
	34	++++	-	N.D.[5]
	35	N.D.	+	Infectious[6]
	38	++++	-	6/6 marmosets
	41	+	-	N.D.
	47	-	+	4/6 marmosets
Cheek	34	++++	-	1/6 marmosets

Footnotes: [1]Days after inoculation of volunteer
[2]Presence of particles when studied in the laboratory of Dr. Robert Purcell, NIH, see reference 7
[3]Presence of fecal antigen when studied in our laboratories by Dr. Geoffrey Cross, see reference 8
[4]Development of hepatitis after the inoculation of 0.5 ml of a 6.25% clarified suspension of feces intramuscularl
[5]N.D.= not done
[6]This stool was centrifuged on a cesium chloride gradient (1.13-1.55 gm/ml) in an attempt to band infectivity, as has been done with serum and plasma (see Table 2). Each fraction contained infectivity ("smearing") and altogether 32 of 48 inoculated marmosets developed hepatitis.

Table 10: Tests for fecal antigen on coded specimens sent by Prof. A. Zuckerman (8-21-74)

Sample Number	IEOP Titer	Gel Diffusion*	Interpretation	Origin of Specimen
1	null	-	negative	Hepatitis, acute
2	≥32	+	positive	Hepatitis, acute
3	4,?16	-	negative	Control
4	≥32	+	positive	Hepatitis, acute
5	≥32	+	positive	Hepatitis, acute
6	16	-	?	Hepatitis, acute
7	4	-	negative	Control
8	≥32	-	negative	Control
9	≥32	+	positive	Hepatitis, acute
10	4	-	negative	Control
11	≥32	+	positive	Hepatitis, acute
12	16	-	?	Hepatitis, acute

*Line of identity with standard fecal antigen
Specimens 6 and 12 showed some deflection of the line, but not a true line of identity

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